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Hydrodynamic behavior of Human Whole Salivary Mucin: A Dynamic Light Scattering study

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Abstract:
Human salivary mucin is a high molecular weight, a primary mucous membrane coating glycoprotein sharing common features with mucin found in other body mucosal surfaces, these features includes a protein core and carbohydrate side chains, forms the first line of defense against microbes. Salivary mucin modulate oral micro flora by attaching and proliferating favorable microbes and clearing pathogens. This function is attributed to the complex formation between mucin subunits and non-mucin species resulting into formation of supra-structures that are stabilized by covalent and non-covalent bonds. These structures are shown to be responsible for high elasticity, adhesiveness and low solubility. Mucin also protects oral hard and soft tissue from adverse conditions through sol- gel phenomenon by reduction and increase in hydrodynamic radii respectively. Thus, architecture of mucin play an important role in protection of oral cavity so this study focuses on study on effect of acidic and basic pH, detergents (like CHAPS, TRITON X -100, SDS), organic solvent (ethanol) and reducing agent (β - mercaptoethanol) on the hydrodynamic radii of purified mucin of human whole saliva, which is a primary parameter to study sol- gel phenomenon. Mucin was purified using Sepharose CL – 4B chromatography and Caesium Chloride density gradient ultracentrifugation. Hydrodynamic size distribution was measured using dynamic light scattering. It was observed that folding and unfolding of the protein core was due to increase in hydrophobic interactions, accomplished by change in hydrodynamic radii. The detergent like SDS forms complex with Mucin and reduces its solubility. Mucin was also subjected to nicotine and was found to have a deleterious effect on it as evident on reduced hydrodynamic radii of mucin as compared to the normal, postulating a probable mechanism of tobacco to overcome the protective function of Mucin, the earliest step towards the carcinogenesis.

Keywords: Mucin, DLS, CHAPS, SDS, Nicotine.

Introduction

Mucin is very high mol. Wt. (of order of 10^6 Dalton) glycoprotein present in all mucous secretions. It primarily plays role in protection of epithelial cells [1]. Mucin glycoprotein has common structural elements consisting of protein core and oligosaccharide side chains linked to serine and threonine residues [2]. Mucin from various secretions shares these common features but differ in complexity due to their saccharide chains, polypeptide backbone and physical properties [3]. Mucin structure can be deduced to two regions, namely rod-like segments, which are substituted by oligosaccharides, scattered with extra flexible and non-glycosylated stretches of the protein core [4]. Mucin coats the gastrointestinal, genito-urinal and respiratory tracts as well as the oral cavity. Mucin in any of its site is present heterogeneously and not as unimodal polydispersed distribution [5]. In the mouth it is in form of saliva, where it lubricates and protects the oral cavity and provides a non-specific immune defense through mucin [6]. The biological relevance of this property of mucin is not fully understood, but one possibility is that they act as "decoy "receptors for the prevention of the binding of pathogens to epithelial cells [7]. The total concentration of mucin in saliva is approximately 200 µg/mL [8]. It was studied by electron microscopic studies of human mixed saliva that a minimum concentration of mucin is required to form a filamentous network structure with colloidal characteristics [9]. MUC 5B is the major mucin present in salivary mucus [10]. Mucins are also rich in albumin [11], and the other components associated with salivary gel matrix are secretory immunoglobulin A, lactoferrin, lysozyme, MUC 7 and agglutinin [12].

There are many subtypes of secretory mucins, of which MUC2, MUC5AC, MUC5B, MUC6 and MUC11 are gel- forming; MUC7, MUC8 and MUC9 are non-gel forming; MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC16, MUC17 and MUC20 are membrane bound; and MUC14, MUC15 and MUC18 are unclassified [13]. Unlike the gastrointestinal mucin, which is secreted in water-insoluble form, the salivary mucin is secreted as viscous soluble form [14, 15]. The previous reported studies on porcine gastric mucin showed that it undergo an increase in viscosity at low pH [16]. The dynamic light scattering (DLS) studies of porcine gastric mucin showed a decrease in diffusion co-efficient with decreasing pH, suggesting an apparent increase in size. Hydrophobic dye binding studies with 1-aniline – 8- naphthalene sulfonate (ANS) reiterated a conformational change of mucin from random coil at pH ≥4 to an extended conformation at pH< 4 due to exposure of hydrophobic binding sites at low pH [17]. This phenomenon of gelation is supposed to protect the gastric mucin at low pH, although the phase behavior of mucous layer is in ambiguity and actual mechanism of gelation is not fully understood [18]. It has been proposed in a study on cervical mucin that in addition to entanglement [19], there are specific lectin- like regions which may be responsible for gel properties of mucin [20]. It is also noted that hydrophobic regions in mucin are responsible for there complex formation and sticky character [21, 22]. Urea has shown to unfold the pig stomach mucin to produce more extended three-dimensional configuration [23]. Interestingly, chelators like EDTA did not have any effect on the viscosity of pig gastric mucin [24].

We undertook the task of studying the effect of various pH, detergents (CHAPS, TRITON X -100, SDS), reducing agent (β – mercaptoethanol), organic solvent (ethanol) and carcinogen (like nicotine) on human whole salivary mucin by observing hydrodynamic radii of mucin by Dynamic Light Scattering which may help in future to predict the possible reason of sol - gel phenomenon and mucin as a physical barrier to the environment changes in oral cavity. Change in the physical state of mucus is already shown to be responsible for various diseases of oral cavity. Hydrodynamic radius, which is one of the biophysical properties of mucin upon which sol-gel phase depends and any change in size, can alter its physiological function.

Material and Methods

Sample collection:

Unstimulated whole human saliva (about 5 mL per subject) was collected from 10 healthy donors in a tube placed in a beaker filled with ice packs. Collection was done 2 hour after meals on the day of experiment as described earlier [25], and pooled in presence of protease inhibitors like 1 mM phenylethanesulphonylfluoride (PMSF) and 5 mM benzamidine hydrochloride. The pooled saliva was clarified by two cycles of centrifugation at 8000× g for 10 min at 4 °C to remove epithelial cell debris. The salivary supernatant was stored at -20 °C until use.

Mucin Purification:

Mucin Solubilization

The supernatant was subjected to overnight solubilization by with 6 M Guanidinum Hydrochloride (GdnHCl) in the presence of 1 mM PMSF and 5 mM benzamidine hydrochloride to remove the proteins associated [26], at 4° C on magnetic stirrer. The insoluble mucin was further separated by centrifugation at 4400× g at 4° C for 15 min.

Sepharose CL-4B Chromatography

The supernatant was further purified by Sepharose CL 4B (GE Healthcare) chromatography. The column 1.6cm× 120 cm was loaded with 2 mL of the clear mucin solution and eluted with 4 M GdnHCl as described previously [27]. The flow rate was kept at 0.1 mL/min and the experiment was carried out at 25 ° C. The protein content was measured at 280 nm and the void volume of the column was pooled.

Cesium Chloride (CsCl) Density gradient ultracentrifugation

CsCl Density gradient ultracentrifugation (Beckman Ti45 rotor) was used to further purify the sample obtained from the void volume of sepharose CL- 4B column in two step procedure described previously [27, 28]. For the first step, the sample had 4 M GdnHCl and initial density was kept at 1.4 mg/mL and centrifuged at 40000 cycle/ min for 70 hours at 15 ° C. In the second step of the procedure the density was kept at starting density of 1.5 mg/mL with sample adjusted to 0.2 M GdnHCl at same temperature and time. The protein content was measured at

280 nm wavelength. The purified sample was destined to DLS to verify its homogeneity.

Sample preparation for Dynamic Light Scattering:

Mucin concentrations used for experimentation were 5mg/mL, based on lyophilized weight. The sample solution was in presence of 6 M GdnHCl to maintain the mucin solubility.

For studying the effect of pH the salivary mucin was dissolved in 50 mM Glycine HCl buffer (pH 3), 50 mM Sodium Acetate buffer (pH 5), 50 mM Phosphate buffer (pH 7) and 50 mM CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid) buffer (pH 9). 1% of nicotine (Sigma, USA) at various pH adjusted with the above buffers was also used for studying its effect on mucin.

Detergents in concentration of 5, 10 and 20 % of CHAPS (zwitterionic), TRITON X-100 (non –ionic), and SDS (anionic) were used for DLS experiments. 5, 10, 15 and 20% Ethanol (organic solvent) were used to study their effect on mucin. 0.5, 1, 2 and 5 % of β- Mercaptoethanol (reducing agent) was also used for its effect on whole salivary mucin.

Mucin samples were always centrifuged at 8000 rpm for 15 min immediately prior to light scattering analysis to remove any residual dust; the resulting nearly clear solutions were filtered by gravity through 0.22µm Millipore filters.

Dynamic light scattering studies:

Theory of Dynamic Light Scattering

Dynamic light scattering is based on two assumptions, first, that particles undergo the Brownian motion (or the "random walk"), with larger particles moving more slowly. In this situation the probability density is given by:

$$P(r,t/0,0) = (4 \Pi Dt)^{-3/2} \exp(-r^2/4Dt) - (1)$$

Where, D is the diffusion constant.

The second assumption, particles in the experiments used are of the size smaller than the molecular dimensions. Hence, we can apply Stokes – Einstein equation that and from which we can derive a formula that easily gives diffusion constant:

$$D = k T/ 6\Pi\eta a \quad -- (2)$$

When the laser is passed onto the collimator lens and hits the cell with solution, the light is scattered and picked up the photomultiplier that transforms the intensity variation into voltage variation. By comparing the fluctuations in intensity of scattered light at an initial time with subsequent measurements at later times, measuring the fluctuations for short period and then multiplying them by the fluctuations at the later period gives us a correlation of how the signal compares over time.

Since the movement of each particle is independent of one another it gives a frequency spectrum of intensity of scattered in form of a Lorentzian shaped line whose width depends upon the diffusion constant D and scattering angle:

$$S(\omega) = \Gamma(\theta)/ [\omega^2 + \Gamma 2 (\theta^2)] \quad -- (3)$$

Where $\omega = 2\Pi f$ with f is the roll-off frequency.

$$\Gamma(\theta) = 2D \{[4 \Pi (\lambda/n)] \sin ½ \theta\}^2 \quad -- (4)$$

Where λ is the wavelength of the incident light, n is the refractive index of the medium and θ is the scattering angle.

By shifting the data by small interval τ, we can see how the pattern correlates. Eventually as the particles move around, there is no correlation between the current fluctuation pattern and the original one. By applying WIENER – KHINTCHINE theorem for random process, it gives spectrum through co-relation function. Spatial co-relation can transform into phase co-relation according to:

$$S(\omega) = e^{i\omega t} R(\tau) d\tau \quad -- (5)$$

Half the width at half the Lorentzian line is correlation time τ, which depicts the time required for the particle to come out of the phase. Hence,

$$\tau = \Pi/DK2 \quad – (6)$$

$K = 4\Pi (\lambda/n) \sin(\theta/2)$

Therefore, D can be calculated as,

$$D = k b T/ 6\Pi \eta a \quad -- (7)$$

The rate of the exponential decay is inversely proportional to the size of the particles because smaller particles move faster. From the decay

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rate it is possible to calculate the hydrodynamic radius (a) using Stokes's Einstein equation.

DLS studies were performed on samples mentioned above which were injected manually into the flow cell (30µl) and illuminated by 100mW, 660nm laser diode using RiNA LASER spectroscatter 201. PMgr v3.01p17 software supplied with the instrument was used for data analysis.

The change in size of mucin formed by various agents was deduced from correlation functions generated by fluctuating intensity of light scattered by solution of mucin.

Results:

Mucin preparation from Saliva

Mucin was purified using sepharose CL 4B column chromatography, Figure (1A). The void volume of the column was pooled and further purified using two step Caesium Chloride Density gradient ultracentrifugation, Figure (1B) as described previously [27,28].

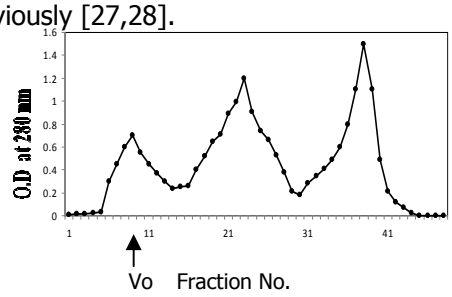

FIGURE (1A): Sepharose Cl-4B column chromatography of crude human saliva. 10 mL of whole human saliva was loaded onto 1.6 × 120 cm column equilibrated and eluted with 4M GdnHCl at flow rate of 0.1 mL/min.

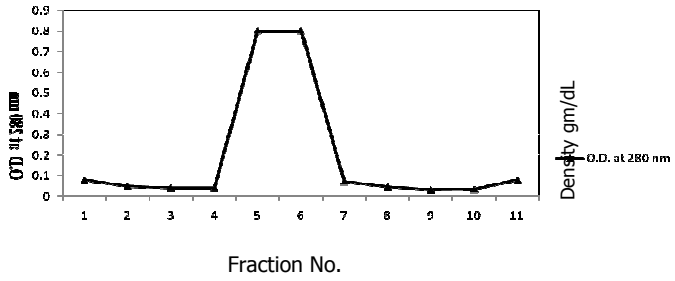

FIGURE (1B): CsCl Density gradient ultracentrifugation. Void volume (Vo) from Cl- 4B sepharose column was set to density of 1.5 gm/dL with caesium chloride. 5mL tubes were centrifuged at 300000 × g for 24 hours and fraction 5 and 6 from the density between 1.4 to 1.5 gm/dL were pooled.

Dynamic Light Scattering Analysis

The fractions 5 and 6 of the density gradient ultracentrifuge were pooled and subjected to DLS. A typical auto correlation function graph for human whole salivary mucin was obtained, figure (2A) and figure (2B) shows the hydrodynamic size distribution of the mucin fragment mostly lying in the range of 90 nm after purification. The CUMULANT ANALYSIS on purified mucin obtained from 10 experiments was performed to avoid incoherency of the procedure, figure (2C).

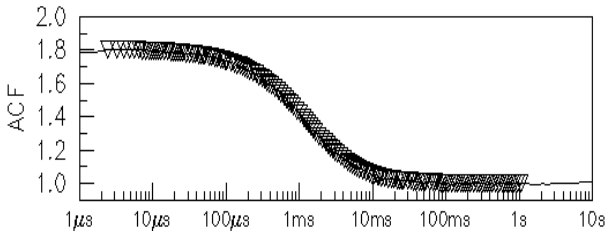

FIGURE (2A): A typical autocorrelation plot for the purified mucin solution (5mg/mL).

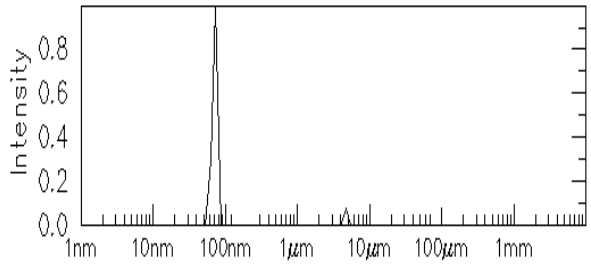

FIGURE (2B): Shows the peak around 90 nm for purified mucin solution in 6M GdnHCL.

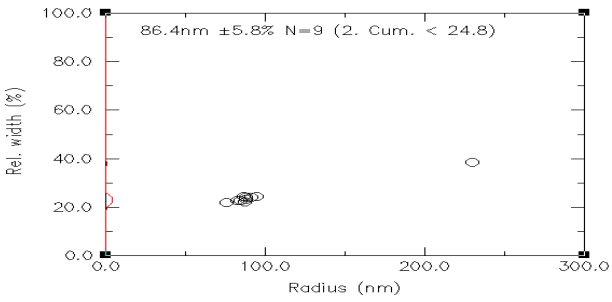

FIGURE (2C): Shows the CUMULANT ANALYSIS on 10 sets of purified mucin solution in 6 M GdnHCl (5mg /mL) containing fragments in the range of 90 nm.

We have examined the pH-induced changes in aggregating properties of whole salivary mucin. A reciprocal relation was observed between the hydrodynamic radius and the pH. Hydrodynamic radius was larger (124.21 nm) at pH 3 than at pH

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7(86.66 nm) and pH 11 (21.7 nm) as highlighted in figure (3).

Mucin when subjected to 1% nicotine at various pH showed to decrease hydrodynamic radii more than what pH alone could have caused as shown in figure (3). The hydrodynamic radius of mucin in presence of 1% nicotine was less than it should be at a given pH. There was a steady decline in hydrodynamic radius from 101.4 nm at pH 3 to 21.7 nm at pH 11.

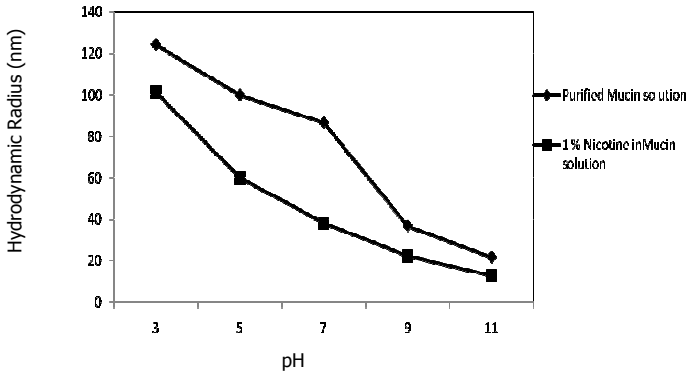

FIGURE (3): Decreasing hydrodynamic behavior of nicotine on mucin irrespective of pH changes.

Detergents like CHAPS (zwitterionic detergent), TRITON X-100 (non – ionic detergent) and SDS (anionic detergent) disaggregated mucin to a greater extent when compared with native mucin, depicted in figure (4).

A higher hydrodynamic radius of mucin was exhibited than native reflecting higher degree of aggregation that increases proportionally on increasing concentration of ethanol as shown in figure (5).
Mucin treated with 0.5, 1, 2 and 5 % β - mercaptoethanol exhibited a trend of reducing hydrodynamic radius, in figure (6).

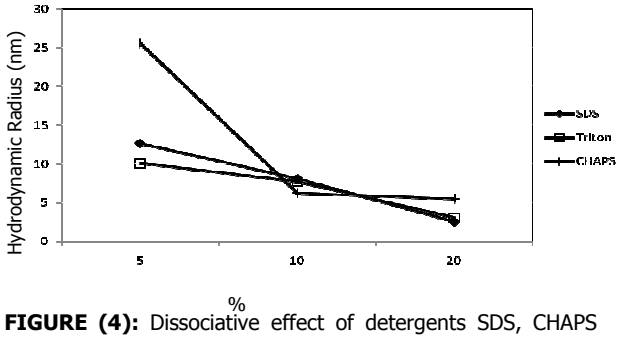

FIGURE (4): Dissociative effect of detergents SDS, CHAPS and TRITON X-100 on mucin solution.

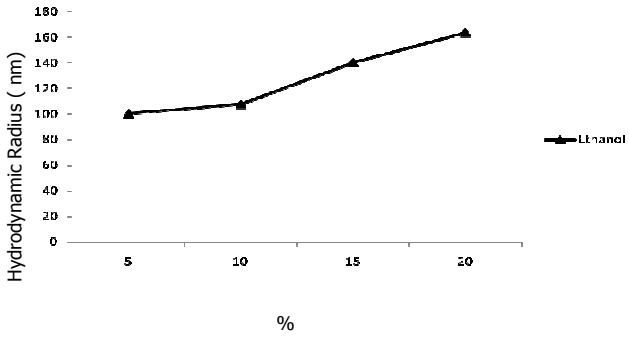

FIGURE (5): Effect of Ethanol on mucin in terms of altering hydrodynamic radii.

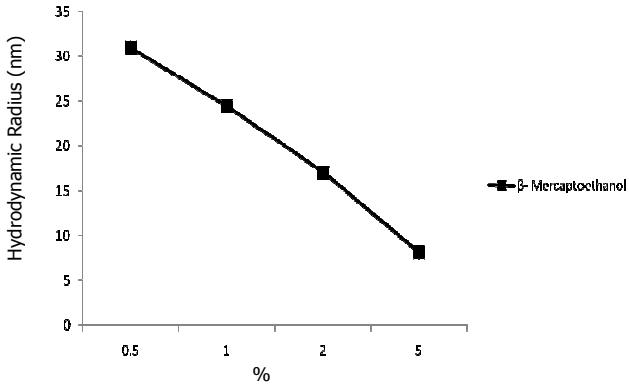

FIGURE (6): Effect of β- mercaptoethanol on hydrodynamic radii.

Table 1 summarizes the effect of various agents on hydrodynamic radii and diffusion constant of purified human whole salivary mucin.

Discussion:

It was observed on varying pH that the hydrodynamic radius of mucin followed inverse relation. As the pH was reduced the hydrodynamic radius of mucin increased. This suggests that hydrophobic residues may be exposed at low pH that resulted in aggregation. The changes could be attributed to the unfolding of the hydrophobic domains at low pH. Studies on Pig Gastric Mucin have already shown extended conformation and high hydrodynamic radii at pH 2 [16]. These observations are consistent with previously reported data on Pig gastric mucin.
Mucin which is known to exhibit a tendency to aggregate and form gels (Taylor et al) which is responsible for visco-elastic and lubricating

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TABLE 1

Summarizes Mucin response to various conditions in terms of changing Hydrodynamic radii and Diffusion constant

	Hydrodynamic Radii (nm)± SE	Diffusion Constant (D)
Mucin Solution (pH)		
3	124.21 ± 0.5	0.23 ± 0.01
5	100.02 ± 0.3	0.29 ± 0.01
7	86.66 ± 0.2	0.32 ± 0.03
9	36.95 ± 0.3	0.38 ± 0.02
11	21.7 ± 0.8	0.43 ± 0.01
Mucin solution + 1 % Nicotine (pH)		
3	101.4 ± 0.6	\0.31 ± 0.02
5	60.11 ± 0.1	0.35 ± 0.02
7	38.1 ± 0.1	0.39 ± 0.01
9	22.43 ± 0.4	0.41 ± 0.03
11	12.85 ± 0.3	0.44 ± 0.02
Detergents (%)		
Triton X -100		
5	10.07 ± 0.2	0.47 ± 0.02
10	7.69 ± 0.3	0.56 ± 0.02
20	3.1 ± 0.3	0.63 ± 0.05
CHAPS		
5	25.58 ± 0.5	0.41 ± 0.04
10	6.24 ± 0.1	0.55 ± 0.01
20	5.51 ± 0.2	0.58 ± 0.01
SDS		
5	12.63 ± 0.1	0.42 ± 0.03
10	8.05 ± 0.1	0.51 ± 0.01
20	2.53 ± 0.3	0.65 ± 0.05
Ethanol (%)		
5	110.21 ± 0.2	0.23 ± 0.01
10	107.7 ± 0.1	0.23 ± 0.01
15	140.2 ± 0.3	0.20 ± 0.02
20	163.53 ± 0.3	0.18 ± 0.01
β- mercaptoethanol (%)	30.95 ± 0.5	0.2 ± 0.01
5	24.43 ± 0.1	0.2 ± 0.01
10	17.05 ± 0.1	0.2 ± 0.02
15	8.1 ± 0.2	0.3 ± 0.01
20		

properties. 1 % nicotine showed a decrease in hydrodynamic radii of the whole salivary mucin compared to non-nicotine treated sample. This may be one of the physical factors by which nicotine act on salivary mucin and may hamper its gel forming property. Thus, nicotine may be one of the primary factors responsible for deleterious effect of tobacco on mucin.

It is of some practical interest to determine how detergents affect mucin since some detergents find important role in processing food. This is clearly seen in DLS measurements as a significant decrease in hydrodynamic radius of mucin with increase in SDS concentration. This has already been shown in earlier studies [31] that mucin form complex with SDS resulting into reduced surface affinity and increased water solubility. The more densely packed native aggregate structure has a higher susceptibility to detergents in general. However, none of the studies is available to our knowledge that depicts role of TRITON X-100 and CHAPS on mucin and needs some further studies in future.

The solvation layer around mucin may be decreased as the ethanol progressively displaces water from mucin surface and binds it in hydration layer around ethanol molecules. With smaller hydration layers, mucin may aggregate by attractive electrostatic forces. The organic solvents like ethanol decrease dielectric constant of water, which allows mucin molecules to come closer, thus, forming aggregation. No studies are available in literature citing effect of ethanol on mucin. There is no change in diffusion constant upon increasing ethanol concentration suggested there might not be any conformational change rather just self –association of the mucin fragments.

β –mercaptoethanol, a known reducing agent lyse disulfide bond, this disintegrates mucin into smaller fragments and hence, reduction in hydrodynamic radius. This is related to the fact that mucin has disulfide linkages to its credit providing a mesh like network of glycoprotein. And also there is no change in diffusion constant, the reduction in size of the mucin fragment may be purely be a response to lysis of disulphide linkages then to altered morphology of the mucin.

Conclusions:

Human oral cavity environment is dynamic in nature and largely depends upon the type of food we eat. With the inclusion of diet rich in processed food the human oral mucosa is exposed to variety of agents whose effect on oral cavity environment is largely unknown. The Salivary mucin which is a primary physical barrier protecting oral mucosa through which nutrient interact and diffuse through, in order to absorb and gain access to circulatory system. Biophysical properties of mucin in solution may play important role in acting as a barrier to bacteria and forming muco-adhesive interactions protecting it from harsh physical conditions. An understanding of biophysical properties of whole salivary mucin in presence of various agents will be of considerable interest to design specific improved biophysical mucin molecule in future. This study reports the changes in hydrodynamic

radii of Human whole salivary Mucin at various pH changes, in presence of denaturants, detergents, reducing agent and nicotine as shown in Table 1. However, further studies are needed to know the actual mechanism of gelation and role of mucin as a barrier towards the mutagenesis induced by tobacco. The overall study may help in postulating some of the protective functions of mucin as a pure physical phenomenon and throw some light on mucin as a physical barrier towards various changes in chemical oral environment.

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